Monograph Report

No 9

Assessment of Mutagenicity of Industrial and Plant Protection Chemicals

June 1987

ISSN-0773-6347-9

ECETOC

MONOGRAPH No. 9

BRUSSELS, 29 JUNE 21 1987

MONOGRAPH No. 9

ASSESSMENT OF MUTAGENICITY OF INDUSTRIAL AND PLANT PROTECTION CHEMICALS

European Chemical Industry
Ecology & Toxicology Centre

ISSN - 0773 - 6347 - 9

Avenue Louise 250, Bte 63
B - 1050 Brussels, Belgium

ECETOC MONOGRAPH No. 9

CONTENTS

Page Nos.

A. SUMMARY..... 2

B. PREFACE.....2

C. STRATEGY FOR THE DETECTION OF GERM CELL MUTAGENS..... 4

1. General Considerations..... 4
2. Selection of Assays and Interpretation of Test Results..... 6

D. CORRESPONDENCE OF PROPOSED TESTING STRATEGY WITH EXISTING GOVERNMENTAL REQUIREMENTS.....13

E. RECOMMENDATIONS.....15

F. BIBLIOGRAPHY.....16

G. APPENDICES.....18

1. Definitions.....18
2. Review of Regulatory Requirements for Mutagenicity Testing.....19
3. Members of Task Force.....26
4. Members of ECETOC Scientific Committee.....27

A. SUMMARY

A strategy is proposed for the evaluation of the mutagenic potential of industrial and plant protection chemicals. A minimum of two genetically independent *in vitro* tests is recommended as primary testing procedure.

If negative results are obtained from two tests there is no need for the conduct of additional tests. Testing *in vitro* beyond the two stage systems does in general not give more information concerning "non-mutagenicity"; recent studies show that almost all of the known mammalian mutagenic chemicals can be detected with a combination of the *Salmonella* microsome test and cytogenetic tests *in vitro*.

If positive results occur in one or both of the primary tests, the genotoxic status of the chemical under investigation should carefully be assessed.

A testing strategy based on the use of a complex battery of tests and the generation of multiple data can no longer be justified scientifically.

B. PREFACE

The growing awareness that some chemicals may have mutagenic properties has resulted in regulatory and administrative measures aimed at detecting and classifying mutagenic substances. Industrial and plant protection chemicals to which human beings are exposed, require an appraisal of their mutagenic potential to man. An ECETOC Task Force (TF) was set up to give guidance on mutagenicity testing for these chemicals with the following Terms of Reference :

1. To recommend which tests must be carried out, and in what sequence, to assess the mutagenicity of industrial and plant protection chemicals in germ cells.

2. To assess whether such testing would meet the main regulatory requirements for these chemicals.

Genetic information is stored in the nucleic acids (e.g. DNA, RNA) and the mechanism of duplication and transcription of this information is similar in all living organisms. Therefore, if mutagenic activity is observed even in prokaryotes it is possible that the substance or its metabolite(s) may be harmful to man. Mutagenicity can thus be evaluated in a qualitative way by determining the

genotoxicity of chemicals with various mechanisms of action in organisms involving different complexities of DNA organisation.

In strict scientific terms, a mutagen* is a substance which induces heritable change in the genetic material of cells or organisms. Mutations can be expressed as gene, chromosomal or genome mutations. These changes can be detected by measuring gene mutation and structural or numerical alterations in chromosomes. The detection of covalent binding of a chemical to DNA, or of damage and subsequent repair of the DNA also indicates the possibility of interaction of a chemical with DNA. The simultaneous presence of all types of effects is not necessary for the proof of mutagenic/genotoxic activity* but the use of a battery of tests is needed to detect specific types of alteration. If a mutagenic hazard to man is to be assessed, the route of administration used in the in vivo tests should be relevant to the route(s) of exposure likely to occur in man.

When a chemical to which human exposure occurs is found to be mutagenic, the qualitative and quantitative aspects of its mutagenic activity and the extent of human exposure should all be considered. The likelihood and extent of human exposure depends on the handling practice during production of the chemicals, on its intended use and its occurrence, degradation and persistence in the environment. Since these factors are common to all chemicals, ECETOC considers it justifiable to propose a common strategy to determine genotoxic/mutagenic hazard of plant protection and industrial chemicals.

Mutation can be demonstrated using simple organisms in vitro where there is inheritance of mutagenic events from a cell to its progeny. However, for human hazard assessment, conclusive evidence that a material poses a heritable hazard can only come from mammalian in vivo studies designed to identify germ cell effects e.g. the specific locus, heritable translocation and dominant or recessive lethal tests. Because such studies are protracted, expensive and involve the use of a large number of animals, it is becoming the accepted practice to use evidence from short term tests to predict the potential of chemicals to induce heritable damage. Such evidence may be obtained from studies on somatic mutation and tissue

---

* See Appendix 1 for definitions

distribution (which may e.g. indicate accumulation in the gonads), or from germ-cell mutation studies. These tests are described in Chapter C.

Current practices and recent developments were assessed and a testing strategy for identifying potential mammalian mutagens is proposed. Whether this strategy complies with legislatory requirements was not taken into account at this stage because many of them are under current review. The proposal is for a battery of two simple in vitro tests followed, when necessary, by more informative tests, the type of which is influenced by the results of these initial studies. The basis for the selection of the possible test systems and the proposed strategy is described in Chapter C.

Research has shown that many mutagenic chemicals have carcinogenic activity in mammals and that mutation may be an essential biological event in the development of cancer. Short term tests for the detection of genotoxic activity are therefore generally used also for predicting the carcinogenic activity of chemicals. This Monograph is limited primarily to the assessment of mutagenic activity in germ cells. It is acknowledged that the initial stages of mutagenic assessment and carcinogen screening are common.

In Chapter D and in Appendix 2 the existing legislatory requirements are reviewed and summarised. The EEC and other countries have developed guidelines and schemes for determining mutagenic activity of chemicals during recent years. The OECD has issued guidelines only on the design of a number of mutagenicity tests and is now preparing an introductory paper which will encompass general guidance for such testing but will not prescribe the testing sequence to be performed.

ECETOC is aware that the strategy proposed here is not in complete agreement with all existing legislatory requirements. The present document may provide a stimulus for the refinement of some of the existing regulatory requirements.

C. STRATEGY FOR THE DETECTION OF GERM CELL MUTAGENS

1. General Considerations

The number of confirmed mammalian germ cell mutagens is relatively small, and all have been shown to be mutagenic and genotoxic in most in vitro and in vivo somatic cell assays. It was clear that substances would not be mutagenic solely

to the germ cell, consequently a strong indication of the possibility of germ cell mutagenicity could be obtained from tests performed during screening for genotoxicity and carcinogenicity. Thus, the proposed strategy is based on a hierarchy of testing in which it is implicit that testing may cease at any stage depending on the profile of activities observed. The conclusion that there is activity at any stage of testing provides the stimulus for further testing or leads to an assumption of germ cell mutagenicity. Equally, appropriate negative data generated at any stage of testing can lead to the conclusion that there is no mutagenic hazard to germ cells without the necessity of further testing.

The strategy proposed is shown diagramatically in Figure I. Its aims are :

i. To establish the genotoxic status of the test agent in mammalian cells in vitro.

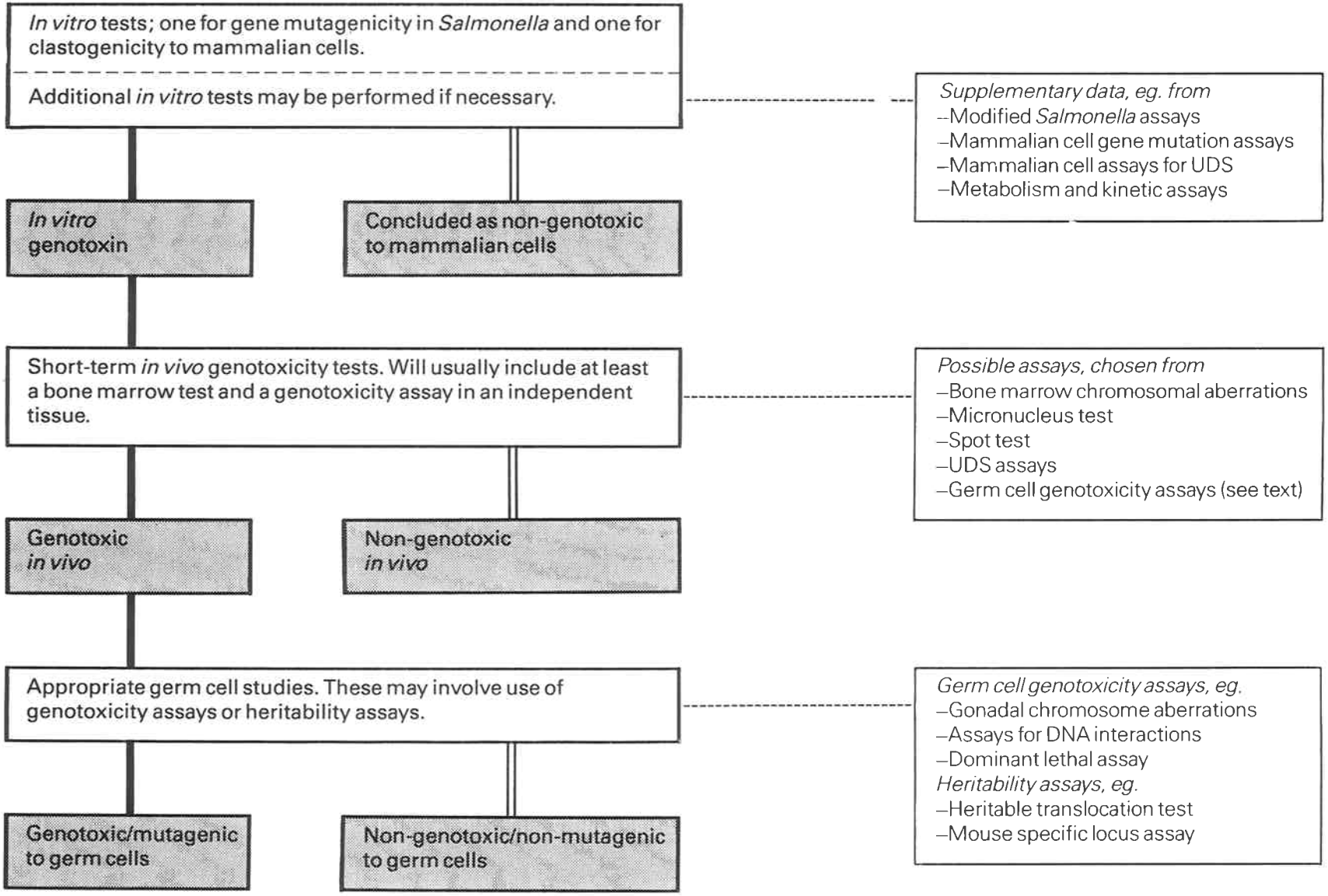
ii. For agents established as genotoxic in vitro, to evaluate their genotoxic potential in vivo.

iii. For agents established as genotoxic in vivo, to evaluate their genotoxic or mutagenic potential in germ cells using genotoxicity or heritability assays.

2. Selection of Assays and Interpretation of Test Results

At the present stage of development of the science of genetic toxicology it was considered inappropriate to make definitive recommendations regarding the selection and conduct of assays. Thus while reference is made in Figure 1 and in the text to, for example, the use of mammalian cell gene mutation assays, no specific test system is recommended. This approach takes account both of the potential value of these tests and of the fact that as a class they are currently under active study in several countries. These studies may lead to changes in recommended test protocols or to recommendations for the use of specific assay systems. Likewise, while present data may suggest that certain test systems do not seem to be optimal for employment in the strategy presented here (e.g. the rodent sperm morphology assay), others which have not been identified here may be appropriate for use under certain circumstances. The object of the following section is to highlight current considerations of relevance to the proposed strategy while leaving freedom for individual scientists to design and conduct experiments appropriate to the particular chemicals under study. For example, if it was evident that an agent was a specific

Fig. 1. – STRATEGY FOR THE DETECTION OF GERM CELL MUTAGENS



clastogen *in vitro* it may be decided to proceed directly to the conduct of cytogenetic assays in the bone marrow and the gonads. This could involve the use of one of the germ cell genotoxicity assays at an earlier stage of testing than would normally be considered. Equally, it may be most appropriate to examine an *in vitro* genotoxin related in chemical structure to an established germ cell mutagen immediately by conducting a germ cell assay. It is implicit in the stategy that positive effects observed in somatic tissues should automatically trigger studies in germ cell assays and that adequate negative evidence in these would be required to conclude the absence of a germ cell hazard. It is emphasised that testing in somatic tissues should cease with the observation of a positive response.

In summary, the proposed testing scheme indicates a general and logical approach to testing which allows deviation in individually justified instances. For example with insoluble substances it may not be possible to carry out *in vitro* tests and therefore tests on somatic cells *in vivo* would be performed at the first stage in such cases.

2.1. Tests In vitro

When determining the mutagenic status of a chemical *in vitro*, several factors must be carefully considered : the number and types of test used, whether genetic specificity of action can be accurately established, how to refer to agents found active and finally, how to respond to isolated positive test responses. In considering these questions the need to minimize testing - but only to a level consistent with the production of a reliable conclusion - must be born in mind. The above four issues are discussed below.

a) *Number and types of test to employ*. There is now general agreement that a minimum of two genetically independent tests should be conducted on each new chemical. Likewise there is general agreement that the primary assay should be the *Salmonella* mutation test and most investigators and legislative guidelines favour the use of an assay for chromosomal aberrations as a minimum second test (Ashby et al., 1985-a). Additional testing may be needed depending, for example, on the responses observed in the two initial tests or because of other information such as established structure-activity considerations. Additional tests will usually be selected from among the following :

i) Mammalian cell gene mutation assays, eg L5178Y ($TK^{+/-}$ /TFT), L5178Y, CHO, V79, ($HGPRT^{+}$/6TG) or human cells such as the TK6 ($HGPRT^{+}$/6TG) assay.

ii) Assays for UDS in hepatocytes or established cell lines.

iii) Assays for SCE in either human lymphocytes or established cell lines.

Some investigators (Gatehouse and Tweats, 1986) favour inclusion of the mouse bone marrow micronucleus assay as a primary screening test. However, it is concluded that the conduct of adequate experiments in vitro is sufficient to identify agents which have the potential to modify DNA structure or function in vivo.

b) Genetic specificity of mutagenic action. Evidence supporting the concept that certain chemicals uniquely produce a certain class of genetic damage in mammalian cells in vitro remains virtually non-existent. If such specificity could be established for a chemical then it would influence subsequent testing in vivo. If genetic specificity is assumed it would be inappropriate to evaluate a specific gene mutagen using a clastogenicity assay in vivo, and vice versa. The data base required to refer to a compound as a specific mutagen may require extensive studies in vitro and these will rarely be done. It is concluded that agents which elicit reproducible genotoxic activity in one or both of the primary in vitro tests should generally be classified as in vitro genotoxins. For practical purposes the subsequent testing strategy does not take into account mutagenic specificity and this is supported by the absence of specific activity in the majority of the known mutagens. In addition, the following factors support the position that short term tests do not necessarily provide evidence for the specific mutagenic activity of a chemical :

i) The activity of an agent as a gene mutagen in the Salmonella assay is influenced by the protocol adopted and the extent of the data generated. For example, an "adequate" experiment according to the OECD criteria could involve the use of only 4 tester strains (TA1535, 1537, 98 and 100) in the standard plate incorporation assay. However, some agents found inactive under such conditions of test may be found active when employing modified test protocols. For example, diethylnitrosamine is only active in the plate incorporation assay when employing elevated levels of S9 fraction in the S9 mix (Müller et al., 1980).

4-Dimethyl-aminoazobenzene is generally inactive in the standard plate assay yet is active when using the pre-incubation test or high levels of S9 fraction in the plate test (Callander, 1986) and several mutagens are almost exclusively active in the new tester strains of Salmonella in use in some laboratories (e.g. TA 102, TA 97).

ii) The activity of a chemical as a clastogen in vitro may be influenced by the cell type or test protocol employed. For example, hexamethylphosphoramide is apparently not clastogenic to CHO cells but is clastogenic to human lymphocytes (Ashby et al., 1985-a,b) and H_2O_2 is clastogenic to separated human lymphocytes but inactive in whole blood (Howard et al., 1985).

iii) The conduct of UDS or SCE assays does not provide data on mutagenicity per se (either gene or chromosomal). Thus data derived from such assays can only be employed to evaluate the genotoxic status of a chemical, in particular they cannot be used to define genetic specificity of action.

iv) A widely used mammalian cell "gene-mutation" assay in current use, the mouse lymphoma L5178Y ($TK^{+/-}$/TFT) assay, has a dual end point. The production of small colonies provides evidence of a clastogenic change to chromosome 11b while large colonies demonstrate gene mutagenicity of the TK locus on this chromosome. Thus, unless small and large colonies are distinguished (which often does not happen), it is inappropriate to associate activity in this assay specifically with gene mutagenicity. A particularly clear case is provided by procarbazine which is not mutagenic to Salmonella but is very active in the L5178Y assay (Clive, 1987). However, its activity in this assay is almost exclusively associated with the production of small colonies. Thus, this "gene-mutation" assay defines procarbazine as a "clastogen".

While the conduct of a minimum battery of standardised in vitro assays can lead to the definition of mutagens in vitro, interpretation of such experiments in terms of genetic specificity of action could be misleading. These several factors combine to indicate that, in the absence of extensive studies, chemicals should not be classified as specific types of mutagen on the basis of observations made in vitro.

c) Terminology associated with activity in in vitro assays. The word mutagen encompasses both gene and chromosomal changes and it is implied that such changes are heritable. A non-specific test such as for SCE or UDS cannot be considered a mutagenicity test and thus their inclusion in a battery complicates use of the word "mutagen" for agents found active in these tests. Further, many of the chromosomal aberrations found in in vitro tests would prove lethal to the cell and do not therefore fulfill the requirement for heritability. These factors, coupled to those discussed in b) above relating to genetic specificity of action, indicate that the term "genotoxic" should be used to describe agents found active in vitro.

d) Handling of isolated positive responses observed in vitro. There are two approaches to in vitro testing, both of which can be supported and both of which have been accomodated by the strategy proposed here. One view is that genotoxic activities observed in vitro with a chemical should be accepted and the chemical be reassessed in vivo for evidence of expression in the whole animal of this innate genotoxicity (Ashby, 1983, 1986). An alternative approach is that, where genotoxic activity is observed only in a single assay, additional mammalian cell in vitro assays should be conducted and that the "weight of evidence" of findings from such tests should be used to define the true genotoxic status of the chemical (Brusick et al., 1986). In both of these approaches agents classified by the investigator as genotoxic in vitro will be assessed further in vivo. Few published data exist at present by which the implied concept of an assay-specific genotoxin can be supported, but it is not an unreasonable concept. The proposed testing strategy is designed to allow for the demonstration of assay specific genotoxins.

e) Metabolism and tissue distribution.
When data on metabolism and kinetics provide evidence that a chemical or its metabolites have reached the germ cells, testing in vivo of a chemical found to be genotoxic in vitro may not be necessary.

In summary, it is proposed that the first step in evaluating an agent for possible germ cell mutagenicity should be the definition of its genotoxic status in vitro using adequate experiments based on a minimum of two genetically independent assays. Agents found to be non-genotoxic following adequate examination in vitro are regarded as unworthy of further study in vivo.

2.2. Tests in vivo.

Two factors endorse the need for experiments in vivo when screening for possible germ cell mutagens : firstly, the expected and observed greater sensitivity of in vitro tests and secondly the need to acquire whole mammal data when screening for a whole mammal event such as germ cell mutagenicity. The first of these factors is associated with the difference between experiments conducted using cultured cells and those involving whole mammals, the second is associated with the relationship between germ cell and somatic cell tissues. Before discussing these two issues it is emphasised that germ cell tests using Drosophila do not have an unique role in screening for potential human germ cell mutagens. This view has recently been endorsed by Vogel who considered that any attempt to use Drosophila germ cell assays as predictors of effects likely to occur in reproductive organs in mammals will fail (Vogel, 1987). In the same document, Vogel outlined a continuing role for Drosophila assays, but only "for predicting mammalian genotoxicity in a broad sense". Thus, assays in Drosophila may go some way towards bridging the gap between assays conducted in vitro and those involving mammalian somatic cells but they have no specific role to play in the prediction of germ-cell specific effects in mammals.

a) The difference between experiments in vitro and in vivo. Experience gained from attempts to predict mammalian carcinogenicity using genotoxicity data generated in vitro indicates that all in vitro test systems are more sensitive than their in vivo counterparts. It means that the activity of an agent in vitro cannot be used to define it as a genotoxin/carcinogen in vivo. The most extensive support for this conclusion comes from recent publications of the US National Toxicology Program (NTP) where the 4 most generally employed in vitro genotoxicity tests (Salmonella, SCE and CA in CHO cells, and the L5178Y assay) have each been shown to detect about one half of the NTP mouse/rat non-carcinogens as positive (Zeiger and Tennant, 1986). It is therefore obvious that in vitro assays can only be employed to predict potential mammalian genotoxins/ mutagens (and carcinogens) and that agents found active in these tests should be evaluated further in whole mammals if reliable predictions of germ cell mutagenicity (and of carcinogenicity) are to be made.

b) The use of in vivo somatic cell assays as a pre-screen for potential germ cell mutagens. One way to detect potential human germ cell mutagens would be to submit all agents shown to be genotoxic in vitro to a mammalian germ cell mutagenicity test. This approach was rejected for two reasons. Firstly, the only assays which provide direct evidence of heritable germ cell mutagenicity (the heritable translocation test and the specific locus assays) demand too many resources for routine use. Secondly, somatic cell mutagenicity data are likely to be available for most chemicals and data can provide strong evidence for and against germ cell mutation.

The proposed testing scheme recommends assessment of the activity of in vitro genotoxins in vivo. Usually the bone marrow test will be used but the results of the preceding in vitro tests may also be taken into consideration on particular occasions. If a negative result is obtained in such a test a second test involving a different tissue may be performed to confirm that the compound is not genotoxic in vivo. In specific cases, reasons may be sufficient to show that the conduct of a second in vivo test is not necessary. This approach is supported by the fact that all rodent germ cell mutagens known to date are also active as clastogens to the bone marrow (Holden, 1982). Holden's observations have remained true with addition of subsequent data : thus the potent new germ cell mutagens acrylamide and ethylene oxide are both clastogenic to the bone marrow (Natarajan and Obe, 1986).

Agents established as genotoxic in in vivo somatic cell mutation tests are classified in the scheme as potential heritable germ cell mutagens. The fact that somatic cell mutagenicity data cannot be taken as conclusive evidence of heritable germ cell mutagenicity is again supported by the study undertaken by Holden (1982) where 18 of 44 bone marrow clastogens were established as inactive in one or more of a range of germ cell genotoxicity/mutagenicity assays. If further data are required for a somatic cell mutagen then two possible courses of action are proposed in the strategy of Figure 1. Either definitive heritable germ cell mutagenicity studies are conducted, or germ cell genotoxicity assays are undertaken. Evidence on germ cell genotoxicity may be derived from the group consisting of the dominant lethal assay, assays for DNA damage in germ cells (using autoradiography (Working and Butterworth, 1984) or whole tissue scintillation counting (Sega et al., 1981)), or cytogenetic tests in cells in the appropriate stages of spermatogenesis. With the possible exception of the dominant lethal assay, data generated using germ cell genotoxicity tests can only be regarded as providing evidence that a confirmed somatic cell mutagen has

reached the testis and interacted with genetic material. Metabolism and tissue distribution data may be used to prove that a somatic mutagen has reached the germ cells. In many cases the difference between such data and data provided by heritable mutagenicity tests may be negligible for predicting mutagenic activity. It is also important to emphasise that, for some of these assays, standard protocols for determining interactions with genetic material in germ cells have yet to be agreed. The only possible weakness of the proposed strategy is that it does not take specific account of chemicals which may uniquely interfere with the metaphase spindle and thereby produce numerical mutations (aneuploidy). However, this is not seen as a significant omission because the known spindle poisons and modifiers (e.g. vinblastine, diethylstilboestrol, etc) are also active as clastogens in vitro and as micronucleus inducing agents in the bone marrow in vivo. In addition, at present no generally available and adequately validated assays for aneuploidy exist (Dallarco et al., 1986).

The sciences of genetic toxicology and molecular biology are developing rapidly, and new techniques, when appropriately validated, should be considered for use. For example, molecular dosimetry techniques appear to offer the opportunity to measure quantitatively the interaction between chemicals and gonadal DNA and this could prove to be of significant value if appropriately validated.

D. CORRESPONDENCE OF PROPOSED TESTING STRATEGY WITH GOVERNMENTAL REQUIREMENTS

A review of the most important regulatory requirements for mutagenicity testing is given in Appendix 2. It should be emphasised that regulatory authorities in general require mutagenicity tests for both carcinogenic and mutagenic hazard assessment.

Most countries require the submission of data from a battery of tests. This battery of tests is however often not precisely defined. The number of tests required varies between countries and there are also differences concerning the genetic endpoints to be tested. Although types of tests to be performed are clearly stated in most cases, only one regulatory proposal exists concerning the interpretation of the results (EPA/FIFRA, 1982).

ECETOC does not see any need for further testing in those cases of negative results in both primary tests. Where positive results are obtained in one or both of the two primary tests, the proposed strategy is essentially in line with most of the current national regulatory practices.

USA/EPA - FIFRA (Draft)(1982)
An extensive battery is proposed with different kinds of tests covering gene mutations, structural chromosomal aberrations and primary DNA damage. The criteria for mutagenic and non-mutagenic chemicals are given. Additional data are required in cases of inconclusive results.

NETHERLANDS/ Pesticides (1981)
The results of at least two tests are needed for a new registration. An in vitro cytogenetic study is requested for all compounds being registered or re-registered.

UK - Control of Pesticide Regulations (1986)
In the section on "Experimental Data", there is an invitation to submit mutagenicity data under "Supplementary studies". However, the tests required are not prescribed but it is anticipated that negative results from the Salmonella (or E. coli) mammalian - microsome test and chromosome analysis in vitro would be the minimum required to conclude that a chemical was not mutagenic. If positive results were obtained it is anticipated that further in vivo tests would be required to clarify the issue and provide a basis for risk assessment. The Pesticide Registration and Surveillance Department would probably accept the proposals of the TF in their present form which are in accordance with the UK-DHSS (1981) guidelines.

ITALY/Pesticides (1978)
The performance of five tests is required. The requirement goes far beyond the ECETOC proposal.

JAPAN/MAFF (1985)
Initially, three in vitro tests providing information on gene mutation, structural chromosomal aberrations and primary DNA damage are required. When further examination is considered necessary based on the results of these studies, appropriate in vivo studies should be selected. While there is a fundamental difference in the initial approach, the requirement for additional studies show more accord with the strategy proposed in this Monograph.

EEC/Industrial Chemicals (1979, 1984)
The EEC "Base-set" includes one bacterial and one non-bacterial "mutagenicity" test. A Salmonella microsome test together with a non-bacterial test e.g. a cytogenetic in vitro test would be acceptable. In place of a cytogenetic in

vitro test a micronucleus or cytogenetic in vivo test could also be performed. Positive results in either may trigger further studies on mutagenicity which would include both in vitro and in vivo assays to provide further information on point mutation and chromosomal damage. The proposed strategy in this Monograph is in accord with the requirements of the EEC 6th Amendment of the Council Directive of 1979 (EEC, 1979).

USA/EPA-TSCA (1985)
No guidance is given but a selection of tests is recommended which will provide information on gene mutation, chromosomal damage, DNA damage and repair and numerical abnormalities.

E. RECOMMENDATIONS

ECETOC recommends that regulatory authorities should be encouraged to amend and reduce their requirements for mutagenicity tests and to recognise that appropriate negative results in the Salmonella microsome assay and cytogenetic in vitro tests are usually all that is required to demonstrate the lack of mutagenic activity of a test substance.

F.BIBLIOGRAPHY

- Ashby, J. (1983). The unique role of rodents in the detection of possible human carcinogens and mutagens. Mutat. Res., 115, 117.
- Ashby, J. (1986). The prospects for a simplified and internationally harmonised approach to the detection of possible human carcinogens and mutagens. Mutagenesis, 1, 3.
- Ashby J., De Serres F.J., Draper M., Ishadate Jr. M., Margolin B.H., Matter B. and Shelby M.D.(1985-a). In Ashby and De Serres (eds). Overview and conclusions of the IPCS collaborative study on in vitro assay systems. Progr. Mut. Res. V.5, Elsevier Publ. Comp..
- Ashby, J., Richardson, C.R., Lefevre, P.A., Callander, R.D. and Styles, J.A. (1985-b). Chloroacetamide-N-metholol : an example of an in vitro and in vivo clastogen which is non-mutagenic to Salmonella. Mutat. Res., 156, 19.
- Brusick, D., Ashby, J., de Serres, I.J., Lohman, P., Matsushima, Y., Matter, B., Mendelsohn, M. and Waters, M.(1986). Weight of evidence scheme for evaluation and interpretation of short-term results. Progress in Clinical and Biological Research, 209B, 121.
- Callander, R.D.(1986). Observed convergence of the Salmonella plate and pre-incubation assays when employing varying levels of S9. Mutagenesis, 1, 439.
- Clive, D.(1987). Activity of procarbazine in the mouse lymphoma L5178Y (TK +/-) mutation assay. Mutagenesis, in press.
- Dallarco, V.L., Voytek, P.E. and Hollaender, A.(1986). Aneuploidy : ethiology and mechanisms. Basic Life Sciences, Vol. 36, Plenum Press, New York.
- ECETOC (1980) : A contribution to the strategy for the identification and control of occupational carcinogens, Monograph No. 2.
- EEC (1979) : Council Directive of 18 September 1979 amending for the 6th time directive 67/548/EEC on the approximations of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Annex VII. Off. J. of the European Comm., L 259, 22, 10.
- EEC (1984) : Commission Directive of 25th April 1984 adopting for the 6th time Council Directive 67/548/EEC on the approximations of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Part B. Methods for the determination of toxicity. Off. J. of the European Comm. , L 251, 271, 1.
- EPA/FIFRA (Nov. 1982). U.S. Environmental Protection Agency, Office of Pesticide and Toxic Substances, Washington D.C. 20460. Pesticide Assessment Guidelines, subdivision F, Hazard Evaluation : Human and Domestic Animals by Office of Pesticide Programs, Draft. EPA 540/9-82-025, PB 83-153916. Organisation and Philosophy of Subdivision F.
- EPA/TSCA (1985). U.S. Environmental Protection Agency. Toxic Substances Control Act Test Guidelines; Final Rule, Fed. Reg. 50 (188), Friday Sept. 27, 1985, 39252.
- Gatehouse, D. and Tweats, D.(1986). Letter to the Editor, Mutagenesis, 1, 307.
- Holden, H.E.(1982). Comparison of somatic and germ cell models for cytogenetic screening. J. Appl. Toxicol., 2, 196.
- Howard, C.A., Richardson, C.R. and Barber, G.(1985). The chromosome damaging potential of hydrogen peroxide as assessed in three in vitro cytogenetic systems. Abst. PM16, 4th International Conference on Environmental Mutagens, Stockholm.
- ITALY (1978) : Proposed amendment to the "Regulations concerning Control of Production, Marketing and Sale of Plant Health Products for the Protection of Stored Foodstuff's Presidential Decree".
- JAPAN/MAFF (1985) : Testing Guidelines for the Evaluation of Safety of Agricultural Chemicals. The Ministry of Agriculture, Forestry and Fisheries.
- Müller, D., Nelles, J., Deparade, E. and Arni, P.(1980). The activity of S9-fractions from seven species in the Salmonella/mammalian microsome mutagenicity test. Mutat. Res., 70, 279.
- Natarajan, A.T. and Obe, G.(1986). How do in vivo mammalian assays compare to in vitro in their ability to detect mutagens. Mutat. Res., 167, 189.
- NETHERLANDS (1981) : Application for the Regulation of a Pesticide. Bureau of the Commission for the Registration of Pesticides.
- OECD (1980) : Mutagenicity tests and their role in identifying mutagens and carcinogens. Background document.

- OECD (1981) : Organisation for Co-operation and Development. OECD Guidelines for Testing of Chemicals, 1981, revised May 1983.
- Sega, G.A., Wolfe, K.W. and Owens, J.G.(1981). A comparison of the molecular action of an SN-1 type methylating agent, methylnitrosourea and an SN-2 type methylating agent, methyl methanesulphonate, in the germ cells of male mice. Chem. Biol. Interact., 33, 253.
- Vogel, E.W. (1987). Evaluation of potential mammalian genotoxins using Drosophila : the need for a change in test strategy. Mutagenesis, 2, 161.
- UK-Control of Pesticide Regulations (1986). Published by HMSO.
- UK- DHSS (1981). Dept. of Health and Social Security. Consultative document on guidelines on Toxicity Testing.
- Working, P.K. and Butterworth, B.E.(1984). An assay to detect chemically induced DNA repair in rats spermatocytes. Environ. Mutagen., 6, 273.
- Zeiger, E. and Tennant, R.W.(1986). Mutagenesis, clastogenesis, carcinogenesis : expectations, correlations and relations. Proceedings of the International Conference of Environmental Mutagen Societies (ICEM). Alan R. Liss, New York, 75.

APPENDICES

Appendix 1

DEFINITIONS

Mutagen : "a mutagenic substance is a clearly defined chemical which can generate a mutation in a particular cell or organism. A mutation is the result of an interaction of the substance with genetic material leading to a change of genetic information which can be passed from parent to progeny (from cell to cell and/or from organism to organism)." (ECETOC, 1980).

Mutagenic activity : Potential of an agent to induce mutation.

Genotoxic activity : This term describes the general potential of an agent to induce DNA damage. Genotoxic activity is usually followed by DNA repair and may, but does not necessarily, lead to gene mutation, chromosomal aberrations, SCE formation and various recombination processes.

APPENDIX 2

REVIEW OF THE MOST IMPORTANT REGULATORY REQUIREMENTS FOR MUTAGENICITY TESTING

1. ABBREVIATIONS

S. typh	Salmonella typhimurium
E. coli	Escherichia coli
B. sub	Bacillus subtilis
S. cere	Saccharomyces cerevisiae
S. pomb	Schizosaccharomyces pombe
N. cras	Neurospora crassa
A. nid	Aspergillus nidulans
D. melan	Drosophila melanogaster
mam.	mammalian
cytogen.	cytogenetics
micronuc.	micronucleus
seg.	segregation
recomb.	recombination
aneup.	aneuploidy
UDS	unscheduled DNA synthesis
SCE	sister-chromatid exchange
RL	recessive lethal
CT	cell transformation
HT	heritable translocation
SLRL	sex-linked recessive lethal
DL	dominant lethal

Regulatory authority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome alterations (structural, numerical)	Other genotoxic effects
UNITED STATES OF AMERICA EPA-FIFRA (1982) (Draft)	A battery of tests is required appropriate to address the following three categories of genetic effects : 1. gene mutations 2. structural chromosome aberrations 3. other mechanisms of mutagenicity as appropriate for the tested chemical.	- S. typh (Ames strains) - E. coli (2 strains) - B. sub (2 strains) - Forward and reverse mutation in : S. Cere S. pomb N. cras A. nid D. melan RL - Specific loci forward and reverse mutation in Chinese hamster lung and ovary - Mouse lymphoma	- A. nid - N. cras - D. melan - in vitro mam. cytogen. - mam. cytogen. - in vivo mam. cytogen. - DL in rat or mouse - Mouse H.T.	- Differential in E. coli, B sub. - S. cere-mitotic recomb - A. nid-mitotic recomb - in vitro mam. UDS - Mouse UDS - in vitro DNA alkaline elution - in vitro SCE - in vivo SCE - in vitro, mitotic interfer. mam. - in vitro, micronuc. - in vitro, mam-CT - Sperm morphology - DNA synthesis inhibition - DNA alkylation

2. REGULATROY REQUIREMENTS - PLANT PROTECTION CHEMICALS
(cont. 2)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome alterations (structural, numerical)	Other genotoxic effects
NETHERLANDS (1981)	Battery of at least two tests, one bacterial and one non-bacterial. A list of tests for consideration is given.	- S. typh - E. Coli host mediated assay. - D. melan RL - mam. in vitro e.g. mouse lymphoma or Chinese hamster cells	- in vitro studies (e.g. D. melan SLRL)	- DNA repair - in vitro SCE
UK - Control of Pesticides Regulations (1986)	Recommends a two-stage approach, stage 1 consisting of a bacterial point mutation assay and two tests in eukaryotic systems (either two point mutation assays or one point mutation and one test for the detection of chromosome abnormalities in vitro). It is recommended that consideration be given to make this stage legally binding. If positive results are obtained, further testing would be required in vivo on mammals.	- S. typh. (4 strains) or - E. coli	- in vivo study (e.g. D. melan SLRL) no tests specified	
WEST-GERMANY		required but not specified		

2. REGULATORY REQUIREMENTS - PLANT PROTECTION CHEMICALS

(cont.3)

Regulatory authority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome alterations (structural, numerical)	other genotoxic effects
ITALY (1978)	Battery of 5 tests is required : two tests for gene- or point mutations (S. typh plus one other); two tests for structural and numeric chromosomal mutations (preferably a non-disjunction test + one other); one test for DNA lesions or DNA repair	- S. typ (4 strains) or similar on other prokaryotes or in eukaryotes - D. melan e.g. RL - in vitro somatic cells. - in vivo mam. point mutations (e.g. mouse specific locus or somatic mutation)	- Non-disjunction in eukaryotes (yeasts, Aspergillus). - Non-disjunction in in D. melan - Non-disjunction in mam. male germinal cells in vivo. - Mam. in vivo in bone marrow. - Mam. in vivo in germinal cells - Mam. in vivo in first stage of embryogenesis. - Micronuc. test. - D. melan heritable and non-heritable effects - Mam. in vivo DL F1 translocation. - Non-disjunction in D. melan	- Mitotic crossing over in yeasts or A. nid. DNA repair. - Evaluation of DNA damage by elution technique. - Stimulation of DNA synthesis in mam. somatic cells. - in vitro SCE

2. REGULATORY REQUIREMENTS - PLANT PROTECTION CHEMICALS

(cont. 4)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome alterations (structural, numerical)	Other genotoxic effects
JAPAN/MAFF (1985)	Tests from all 3 categories are required : when it is necessary to carry out further investigations from the data on these assays, any test suitable for detection of genetic toxicity of DL, mouse specific locus, micronuc., SLRL, *in vivo* cytogen. etc. should be carried out.	- Microbial reverse mutation test e.g. *S. typh.* (at least 4 strains) and E. *coli*	- *in vitro* mam. cytogen. (e.g. Chinese hamster cells or human lymphocytes)	- DNA damage and repair in *B. sub*

2. REGULATORY REQUIREMENTS - GENERAL CHEMICALS

(cont. 5)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome alterations (structural, numerical)	Other genotoxic effects
OECD (1981)	Number and sequence of tests unspecified	- S. typh (4 strains) - E. coli (3 strains)	- in vitro mam. cytogen. micronuc.	
EEC (1979)	The substance should be examined in two tests, one of which should be bacterial and one non-bacterial. This is the "Base set" requirement. Tests not specified. Annex VIII says further studies may be required dependent upon the "Base-set" results. (verification studies)			
EEC (1984)	Annex V lists the following tests, presumably as reference to acceptable verification studies.	- A. nid - S. cere - S. pomb - Mouse lymphoma - Chinese hamster - D. melan, SLRL - CT - Mouse spot test - Mouse specific locus	- DL (rodent) - A. nid somatic seg. - S. cere mitotic aneup. - in vivo cytogen. - Mouse HT	- S. cere mitotic recomb. - UDS-mam. cells - SCE-in vitro human lymphocytes or Chinese hamster ovary cells

3. REGULATORY REQUIREMENTS - GENERAL CHEMICALS
(cont.6)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome alterations (structural, numerical)	Other genotoxic effects
UNITED STATES OF AMERICA EPA - TSCA (1985)	Mutagenicity tests required. No guidance is given as to when these tests are required.	- S. typh (at least 4 strains) - E. Coli (3 strains) - A. nid - N. cras - D. melan SLRL - Mam. in vitro e.g. mouse lymphoma and Chinese hamster cells - Mouse specific locus	- in vitro cytogen. - Micronuc. (mouse recommended) - in vivo mam. cytogen. - D. melan HT - DL (rat or mouse) - Mouse HT	- DNA damage/repair tests in E. Coli or B. sub - in vitro mam. UDS - S. cere mitotic gene conversion - in vitro SCE

APPENDIX 3 : MEMBERS OF TASK FORCE

P. Arni (Chairman)	CIBA-GEIGY (Basel)
J. Ashby	ICI (Alderley Park)
S. Castellino	Farmitalia - Carlo Erba (Milano)
G. Engelhardt	BASF AG (Ludwigshafen)
B.A. Herbold	BAYER AG (Wuppertal)
R.A.J. Priston	SHELL (Sittingbourne)
W.J. Bontinck (Secretary)	ECETOC (Brussels)

APPENDIX 4 : MEMBERS OF ECETOC SCIENTIFIC COMMITTEE

I. PURCHASE (Chairman), Director, Central Toxicology Laboratory	ICI (Alderley Park)
M. SHARRATT, (Vice-chairman), Senior Toxicologist	BP (Sunbury)
B. BROECKER, Coordinator, Product-related Environmental Problems	HOECHST (Frankfurt)
L. CAILLARD, Industrial Toxicological Service	RHONE-POULENC (Paris)
H.O. ESSER, Head of Biochemistry, Agricultural Division	CIBA-GEIGY (Basel)
P. GELBKE, Head, Department of Toxicology	BASF (Ludwigshafen)
U. KORALLUS, Medical Director	BAYER (Leverkusen)
C. LUNDBERG, Chief Toxicologist	NOBEL INDUSTRIES (Karlskoga)
H.G. NOSLER, Head, Coord. Centre for Environmental Protection and Consumer Safety	HENKEL (Düsseldorf)
S. PAGLIALUNGA, Head of Industrial Toxicology	MONTEDISON (Novara)
C.L.M. POELS, Envir. Affairs Division	SHELL (den Haag)
C. DE SLOOVER, Head of Medical Department	SOLVAY (Brussels)
W.F. TORDOIR, Head of Occupational Health and Toxicology Division	SHELL (den Haag)
H. VERSCHUUREN, Head, Department of Toxicology	DOW CHEMICAL (Horgen)

Responsible Editor : D.A. Stringer
Avenue Louise 250, Bte 63
B-1050 Brussels, Belgium

D-1986-3001-45